

New tensio-active molecules stabilize a human G protein-coupled receptor in solution

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Abstract Structural characterization of membrane proteins is hampered by the instability of the isolated proteins in detergent solutions. Here, we describe a new class of phospholipid-like surfactants that stabilize the G protein-coupled receptor, BLT1. These compounds, called C₁₃U₉, C₁₃U₁₉, C₁₅U₂₅ and C₁₇U₁₆, were synthesized by radical polymerization of Tris(hydroxymethyl) acrylamidomethane in the presence of thioglycerol, first endowed with two hydrocarbon chains with variable lengths (13–17 carbon atoms), as transfer reagent. C₁₃U₁₉, C₁₇U₁₆ or C₁₅U₂₅ significantly enhanced the stability of BLT1 in solution compared to what was obtained with common detergents. These molecules therefore represent a promising step towards the structural characterization of BLT1 and possibly other membrane proteins.

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1. Introduction

G protein-coupled receptors are versatile biological sensors that are responsible for the majority of cellular responses to hormones and neurotransmitters as well as for the senses of sight, smell and taste [1,2]. Although significant progress has been made within the last few years in dissecting GPCR-mediated signal transduction pathways [3], understanding the mechanisms underlying ligand recognition and signal transduction through the membrane has been hampered by the lack of information at the molecular level. Structural information on the GPCR family is therefore very sparse, with the excep-

tion of rhodopsin for which several crystal structures have been determined [4–6], and therefore appears as the structural prototype of G protein-coupled receptors (GPCRs).

The primary difficulty encountered in the study of membrane proteins such as GPCRs is that of obtaining the protein of interest. GPCRs are usually present at low levels in biological membranes, and it is rare that a single protein species is a major peptidic constituent of the membrane. Furthermore, membrane proteins are naturally embedded in a lipid bilayer, which is a complex and heterogeneous environment. Finally, membrane proteins are generally not soluble in aqueous solution. The need for membrane proteins to be maintained in surroundings that satisfy their high hydrophobicity therefore requires special synthetic systems for *in vitro* work [7]. Unfortunately, reconstituting purified proteins into such systems has proven to be non-trivial (for a review, see [7]). Maintaining membrane proteins in aqueous solutions, in particular in the context of crystallization assays, is therefore of major scientific and biomedical importance. While classical phospholipids naturally associate with membrane proteins without inducing any unfolding, they cannot be used to maintain them soluble because of their own poor solubility in aqueous solutions. To alleviate the major problem of membrane protein unfolding, aggregation and/or inactivation by classical detergents, less aggressive surfactants such as amphipols [8,9] or fluorinated surfactants [10–13] have been designed and are currently developed. Non-ionic fluorinated surfactants as well as amphipols do not solubilize biological membranes but are able to keep membrane proteins that have been extracted using classical surfactants soluble in water. The possibility of using such surfactants to maintain the native fold of the BLT1 receptor in solution is currently under investigation. Despite the promising results obtained with these molecules, the search for surfactant molecules that could stabilize the native fold of membrane protein better than commercial detergents do is still crucial in the context of membrane protein structural analyses.

We have produced a GPCR, the BLT1 receptor, as a recombinant protein in an *E. coli* expression system with yields allowing structural studies to be carried out [14]. BLT1 is one of the two membrane receptors for leukotriene B₄ [15]. Leukotriene B₄ (LTB₄) is a potent activator and chemoattractant for leukocytes and is involved in several inflammatory diseases [16]. BLT1 therefore represents an important target

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Abbreviations: AIBN, α,α' -azobis isobutyronitrile; CD, circular dichroism; cmc, critical micellar concentration; DDM, dodecyl- β -D-maltoside; DP_n, degree of polymerization; GPCRs, G protein-coupled receptors; HDM, hexadecyl- β -D-maltoside; LDAO, dodecyltrimethylamino oxide; LTB₄, leukotriene B₄; TEA, triethylamine; THAM, Tris(hydroxymethyl)acrylamidomethane; THF, tetrahydrofuran

in the context of drug design. Moreover, this receptor can be considered as a typical class A GPCR with a diffusible ligand and the results with this receptor could therefore be extended to other members of this class of receptors. As stated above, structural analyses require BLT1 to be stable in detergent solutions for periods of time compatible with such studies, in particular with crystallization assays. Indeed, any conformational heterogeneity arising from a partial unfolding of the purified receptor could be detrimental to crystallization. To achieve the highest stability of recombinant BLT1 in solution, we tested here the ability of a series of original surfactants to stabilize the recombinant receptor *in vitro* compared to what is usually obtained with common detergents.

2. Materials and methods

2.1. Materials

LTB₄ was purchased from BIOMOL Laboratories. Fos-choline-16 was from Anatrace and thrombin from Sigma. The solvents and chemicals for the detergent synthesis were obtained from commercial sources (Aldrich, Acros Organics, Lancaster (UK)). They were of reagent grade and were used without further purification. 1,6-Diphenyl-1,3,5-hexatriene and carboxyfluorescein were purchased from ACROS and used without further purification.

2.2. Buffers

Buffer A: 100 mM NaH₂PO₄, 10 mM Tris–HCl, 10% glycerol, 4 mM 2-mercaptoethanol, 0.4% SDS, pH 8. Buffer B: 12.5 mM Na-borate, 10 mM NaCl, pH 7.8 containing the detergent and asolectin (1:2 lipid to detergent ratio). All the surfactants were used at concentrations corresponding to 1.5 times their critical micellar concentration (cmc) (see Table 1).

2.3. Detergent synthesis

2.3.1. General methods. Analytical TLCs were carried out on pre-coated Silica Gel 60F-254 plates (Merck) and visualization was performed with UV light (254 nm), ninhydrine spraying and heating (2% in ethanol) and sulfuric acid spraying and heating (2% in ethanol). Flash chromatographies were performed on Geduran Si 60 (40–63 µm) silica gel from Merck. Spectra were recorded using the following instruments. For ¹H, ¹³C and ¹⁹F NMR spectra: Bruker AC 250; for mass spectra: JEOL DX 300.

2.3.2. Synthesis of lipidomimetics. These compounds were synthesized from the thioglycerol as starting material. First, *S*-trityl mercapto glycerol was prepared in high yield by grafting triphenyl methyl chloride onto thiol function of thioglycerol in tetrahydrofuran (THF) at 0 °C in a presence of triethylamine (TEA). Then, the hydrophobic tails were grafted onto the hydroxyl functions of the thioglycerol residue: this was achieved through carbamate bonds by using the chosen carboxylic acid (myristic, palmitic or stearic acid) in the presence of diphenylphosphorylazide and TEA in toluene at 60 °C. The action of diphenylphosphorylazide on carboxylic acid leads to an alkyl isocyanate that immediately reacts with hydroxyl functions of thioglycerol moiety. The protective trityl group was then cleaved in an acidic medium at room temperature (trifluoroacetic acid–dichloromethane

1/1 v/v). Thus, telomerization of Tris(hydroxymethyl)acrylamidomethane in a presence of this hydrophobic thiol molecule as telogen was performed in a THF/methanol mixture at 65 °C under an argon atmosphere, using α,α' -azobis isobutyronitrile (AIBN) as radical initiator. The AIBN concentration in the reaction mixture was roughly ten times lower than the thiol molecule (called telogen) [17].

The number average degree of polymerization is equal to the amount of repeating Tris(hydroxymethyl)acrylamidomethane (THAM) units *n*. With a given transfer reagent, it may vary from one (monoadduct) to several tens, depending on the [monomer]/[telogen] ratio (*R*₀) adjusted through both starting material and experimental conditions [18]. The proportions of monomer THAM and thiol **3a–c** (telogen) used are reported in Table 1. These proportions were chosen taking into account previous results obtained with THAM telomerization and structural relationships between degree of polymerization (DP_n) and supramolecular systems obtained in water (Michel, N., unpublished data). Each experiment was pursued until the complete disappearance of the monomer. At the end of polymerization, the solvents were removed under vacuum, the remaining telomer was solubilized in a small amount of methanol, then dropped in ethyl ether. The precipitate was filtered off and then submitted to a size exclusion chromatography using a Sephadex G25 column and then lyophilized. The DP_n of the macromolecule, was determined in ¹H NMR by comparing peaks area assigned to the terminal methyl signals in the hydrocarbon tails (δ 0.9 ppm, integral 6H) to hydroxyl groups of THAM (δ 5 ppm, integral 3nH).

2.3.3. Determination of the cmc by fluorescence measurements. The cmc of the C_nU_m surfactants was determined from steady-state fluorescence measurement [19]. Measurements were carried out at 25 ± 0.1 °C on a SPEX-Fluoromax 2 fluorimeter (Jobin-Yvon). Fluorescence emission spectra of samples containing 1.6 µM pyrene were recorded using an excitation wavelength of 335 nm. Emission intensities were recorded at λ_1 = 373 nm and λ_3 = 384 nm [19]. For each surfactant, the cmc values were the average of three independent measurements.

2.3.4. Dynamic light scattering. Particles size and polydispersity were measured at 25 °C using a Zetasizer Nano-S model 1600 (Malvern Instruments Ltd., UK) equipped with a He–Ne laser (λ = 633 nm, 4.0 mW). The Stokes radius (*R*_S) of the particles was estimated from their diffusion coefficient (*D*) using the Stokes–Einstein equation $D = k_B T / 6\pi\eta R_S$, where *k*_B is Boltzmann constant, *T* the absolute temperature and η the viscosity of the solvent.

2.4. BLT1 refolding

The unfolded BLT1 receptor was prepared as recently described [20]. The unfolded His-tagged BLT1 in buffer A was loaded again on a Ni-NTA matrix and immobilized at a protein-to-resin ratio of 0.5–0.6 mg of protein/ml of hydrated Ni-NTA agarose, and refolding was achieved by using linear gradient from buffer A to buffer B. Dissociation of BLT1 from the matrix was then achieved with buffer B containing 300 mM imidazole. Unfolded proteins were discarded and the functional receptor further purified by gel filtration chromatography on a Superdex S200 HR column (1.6 × 70 cm) using buffer B as the eluent. The amounts of dimeric receptor were estimated by chemical cross-linking, as described previously [21].

2.5. Circular dichroism measurements

Circular dichroism (CD) spectra were recorded at 22 °C with a dichrograph CD6 (Jobin-Yvon). The spectra are the average of five scans using a bandwidth of 2 nm, a step-width of 0.2 nm and a 0.5 s averaging time per point. The cell path length was 1.00 ± 0.01 mm. BLT1

Table 1
Physical–chemical data of C_nU_m surfactants

Compound	<i>R</i>	<i>R</i> ₀	DP _n	MW (g mol ^{−1})	Yield (%)	cmc (µM ± S.D.)	Diameter ¹ (nm)	Diameter ² (nm)
4a1 C ₁₃ U ₉	C ₁₃ H ₂₇	6	9	2200	75	7.9 ± 0.7	6.2 ± 0.3	10.3 ± 0.4
4a2 C ₁₃ U ₁₉	C ₁₃ H ₂₇	14	19	3850	78	8.1 ± 0.8	8.5 ± 0.2	12.5 ± 0.3
4b C ₁₅ U ₂₅	C ₁₅ H ₃₁	17	25	4960	91	5.5 ± 0.7	9.1 ± 0.3	13.3 ± 0.3
4c C ₁₇ U ₁₆	C ₁₇ H ₃₅	15	16	3440	89	4.9 ± 0.4	8.1 ± 0.3	12.1 ± 0.2

Diameter (1) is the diameter of the surfactant in the buffer used for refolding as measured by dynamic light scattering; Diameter (2) is that of the protein:surfactant complex measured under the same conditions. *R*₀ is the [Monomer]/[Telogen]₀ ratio, DP_n is the number average degree of polymerization of each compound. Cmc means critical micellar concentration (measured in pure deionized water at 25 °C).

concentrations were determined by UV absorption. A molar absorptivity of $5.0 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$ at 270.5 nm [22] was adopted for LTB₄ without any correction for solvent effects. The *R* factor in Fig. 4B is the ratio of the intensity of the LTB₄-associated CD maximum at 270.5 nm in the presence of saturating concentrations in BLT1 to that measured in the absence of receptor.

2.6. LTB₄ binding assays

The LTB₄-associated CD band at 270.5 nm was selected for monitoring the binding (titrations using other CD maxima gave identical results). Successive additions of BLT1 were carried out whereas the LTB₄ concentration remained nearly constant (usually 10^{-8} M ; dilution effects not exceeding 3%). Binding isotherms were obtained by plotting $([\theta] - [\theta]_0)/([\theta]_{\text{max}} - [\theta]_0)$ as a function of BLT1 concentration, where $[\theta]_{\text{max}}$, $[\theta]_0$ and $[\theta]$ correspond to the molar ellipticities, at saturation, zero occupancy and intermediate occupancy, respectively. The binding parameters were inferred from these titration data by analyzing them with the PRISM software (Graphpad Inc.) and considering a set of usual models for describing the interaction.

2.7. Stability assays

The amount of active receptor as a function of time was determined by circular dichroism. The intensity of LTB₄-associated CD band centered at 270.5 nm in the presence of BLT1 was recorded at increasing time values. The intensity of this band is directly related to the amount of LTB₄ bound to the receptor. The experimental conditions for these CD measurements were those described above for the LTB₄ binding assays.

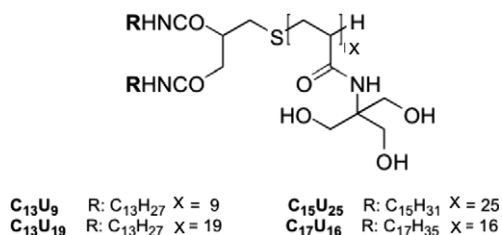


Fig. 1. Chemical structure of THAM derived C_nU_m surfactants.

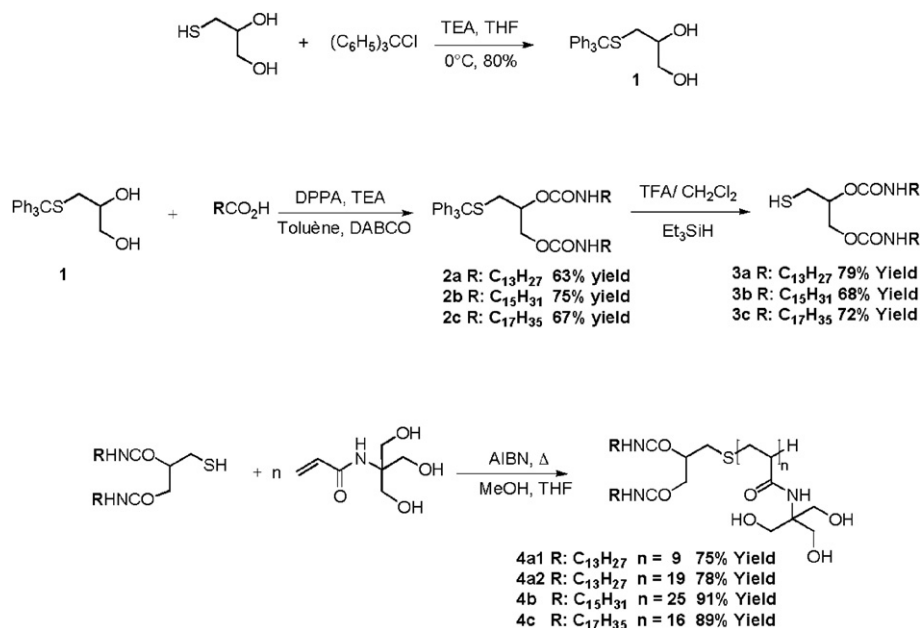


Fig. 2. Synthetic pathway of THAM derived C_nU_m surfactants.

3. Results and discussion

We have combined chemical and biochemical methods to design and test new molecules aimed at stabilizing the BLT1 receptor, and potentially other GPCRs, *in vitro*. Our starting hypothesis was to consider that water-soluble non-ionic surfactants, endowed with two hydrophobic tails, could mimic natural phospholipids, associate with membrane proteins and maintain them soluble in aqueous solution without prohibitive unfolding. We therefore synthesized a new family of synthetic lipids harboring two hydrophobic tails with variable lengths. These hydrocarbon chains were grafted onto the hydroxyl groups of a thioglycerol interface by carbamate groups (Fig. 1). In order to provide a higher solubility in water, the polar head was prepared by radical polymerization of Tris(hydroxymethyl) acrylamidomethane in the presence of this thioglycerol derived transfer reagent [10]. The chemical pathway is described in Fig. 2. Such a chemical pathway allowed us to produce original lipid-like surfactants that make micelles of well-defined size in solution, as assessed by light scattering experiments (the main physical-chemical data of the C_nU_m surfactants, where *n* represents the number of carbon atoms of each hydrophobic chain and *m* the number of Tris motives constituting the polar head – are reported in Table 1). In water these compounds lead to micellar systems that exhibit an average diameter larger than that observed with classic detergents. If this slight increase in the micelle size is a problem for future crystallization assays remains an open question. If this is the case, one could for example envisage the use of small amphiphiles molecules [23] or to modify the nature of the polar head to reduce the micelle size.

The recombinant BLT1 receptor was refolded using the matrix assisted-method we devised [14]. Briefly, the unfolded receptor was bound to a Ni-NTA matrix and then the denaturing detergent (SDS) was progressively replaced by the non-denaturing detergent. The receptor was subsequently recovered from the Ni-NTA matrix and the unfolded fractions

removed by a size-exclusion chromatographic step. The fraction of refolded receptor in Fig. 3A is the ratio of the amount of receptor that is able to bind LTB₄ to that initially bound to the Ni-NTA matrix.

We first tested the ability of the C_nU_m surfactants to increase receptor refolding efficiency. In these assays, we replaced the commercially available detergent that gave the best refolding ratios, i.e. LDAO [14], hexadecyl-β-D maltoside (HDM) [21] or fos-choline C-16 (unpublished data), by C₁₃U₉, C₁₃U₁₉, C₁₅U₂₅ or C₁₇U₁₆. We previously showed in the case of a common detergent, LDAO, that using detergent at high concentrations, i.e. above 2 times its cmc, results in a decreased refolding ratio [14]. A similar behavior was observed with other common detergents such as dodecyl-β-D-maltoside (DDM) (Banères,

unpublished data). Although we did not investigate here the effects of the C_nU_m concentration on the refolding ratio, these compounds were used at concentrations below this 2× cmc limit. In all the cases, the surfactant was used in combination with a mixture of natural lipids from soybean (see Section 2). Like for common detergents (Banères, unpublished data), adding the lipids increased the stability of the refolded receptor (see Fig. 5 for C₁₅U₂₅; a similar behavior was observed with the other surfactants). The fact that lipids are required for an optimal stability of the receptor could seem contradictory with the fact that our surfactants are designed to mimic phospholipids. A possibility would be that some specific lipids are present in the asolectin extract that are required to stabilize the receptor and cannot be mimicked by our surfactant.

As shown in Fig. 3A, no significant change in the refolding efficiency was obtained after refolding the receptor in either C₁₃U₁₉, C₁₅U₂₅ or C₁₇U₁₆ compared to what was achieved in fos-choline-16. In all the cases, a maximal refolding ratio of about 25–30% was obtained. Only C₁₃U₉ gave a dramatically decreased refolding ratio (ca. 8–10%). We previously reported that one of the main factors influencing the refolding efficiency of BLT1 was the length of the alkyl chain of the detergent [14]. Indeed, similar refolding ratios were obtained with different detergents with alkyl chain above C₁₂, whereas decreasing the length of the chain below this value dramatically decreased refolding efficiency [14]. For example, a ca. 10-fold increase in the refolding ratio was obtained when using dodecyltrimethylammonium chloride (LDAO) instead of undecyltrimethylammonium chloride whereas similar refolding ratios were obtained with LDAO and tridecyltrimethylammonium chloride. Such a behavior could explain why the different surfactants produced here that display alkyl chains ranging from 13 to 17 carbon atoms lead to similar refolding ratios.

The functional species in the case of GPCRs are likely to be the dimer [24]. If this is really the case, stabilization of this assembly will be required for analyzing the structural and functional features of the purified receptors. We previously showed that the stability of the BLT1 dimer was highly dependent on the nature of the detergent [21]. We analyzed here the oligomerization state of the receptor refolded in the different C_nU_m surfactants using the cross-linking approach described previously [21]. As shown in Fig. 3B, the dimer is the predominant species whatever the surfactant used is, with the exception of C₁₃U₉ where the refolded receptor appears as a mixture of monomer and dimer in ca. similar amounts. This indicates that all our surfactants besides the one with the shorter polar head, i.e. C₁₃U₉, essentially stabilize the BLT1 dimeric assembly. As stated above, we previously showed that increasing the length of the alkyl chain not only led to higher refolding ratios but also to an increased stabilization of the BLT1 dimer [22]. In agreement with this result, it has also been recently shown that detergents with long alkyl chains, i.e. above C₁₂, lead to a better stabilization of rhodopsin oligomers [25]. The apparent effect of the length of the alkyl chain of the C_nU_m surfactants on the dimer stability is thus in full agreement with our previous observations with common detergents.

We next analyzed the structural and functional properties of BLT1 refolded in the different surfactants described above. BLT1 retains its structural features whatever the surfactant used for refolding is. Indeed, in all the cases, the circular dichroism (CD) spectrum of the refolded receptor in the 200–250 nm region was that of a well-folded protein with a

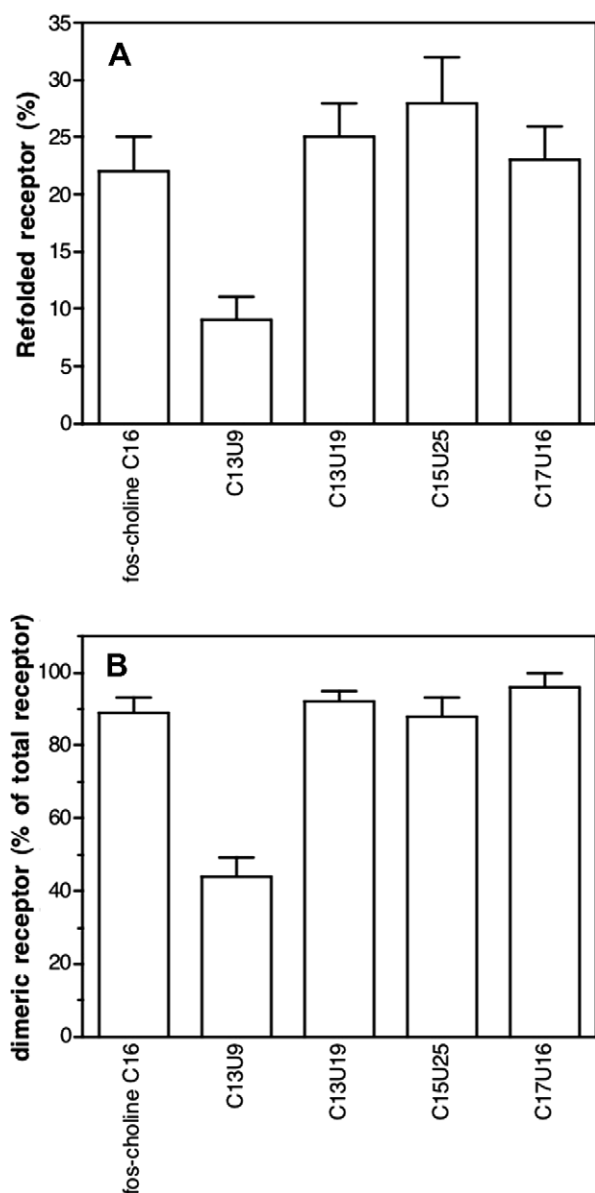


Fig. 3. Refolding and dimerization of the BLT1 receptor. (A) Refolding ratio obtained for BLT1 in different surfactants. The refolding ratio corresponds to the amount of active, ligand-competent, receptor after refolding to the amount of unfolded receptor before refolding (see Section 2). (B) Receptor dimer amount after refolding in the different detergents as determined by chemical cross-linking.

high helical content and similar to that we previously reported for BLT1 refolded in LDAO (not shown). The ligand binding properties of the receptor were assessed by measuring the changes in the dichroic properties of LTB₄ induced by the binding to BLT1. As previously reported [14], free LTB₄ is characterized by a four-band CD spectrum in the 240–290 nm region (Fig. 4A). Binding to the recombinant receptor results in an increase in the intensity of all LTB₄ dichroic bands that can be interpreted as a skewing of the time-aver-

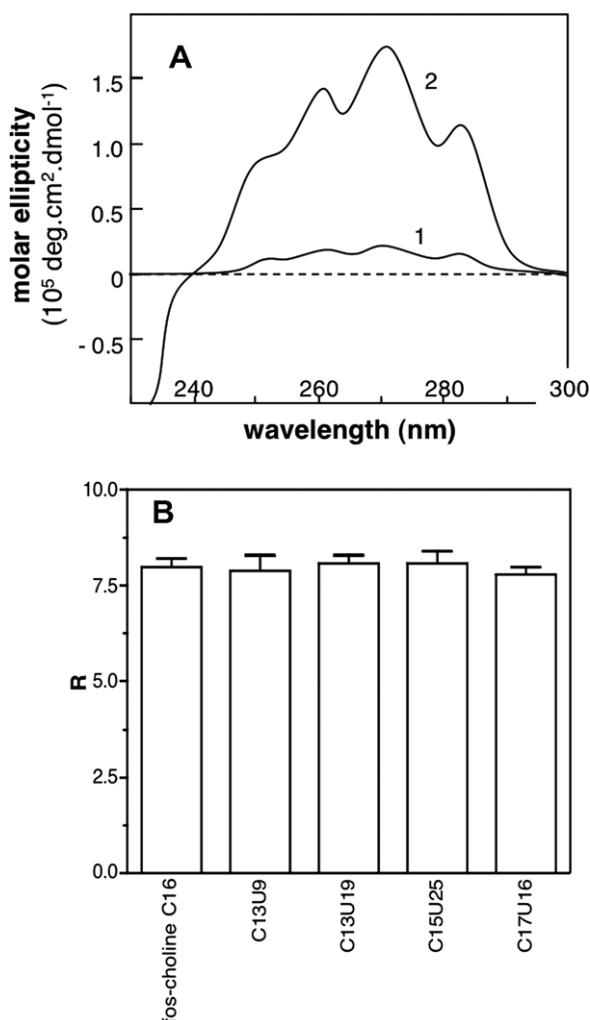


Fig. 4. LTB₄ binding to refolded BLT1. (A) Near-UV circular dichroism spectrum of LTB₄ free (1) or bound to the BLT1 receptor refolded in fos-choline-16 (2). (B) CD increase ratio *R* obtained for the BLT1 receptor refolded in the different detergents. *R* is the ratio of the LTB₄-associated CD intensity at 270.5 nm in the presence of BLT1 to that measured in the absence of BLT1. The error values correspond to the standard deviation from the mean value calculated from three experiments.

aged planar triene in free LTB₄. As shown in Fig. 4B, the increase in the intensity of the dichroic bands of LTB₄ observed in the presence of the purified BLT1 was similar whatever the surfactant used for refolding the receptor was. Even moderate deviations from planarity in the triene motif of LTB₄ cause intense changes in the CD spectra [26]. The changes in the CD properties associated with the triene moiety of LTB₄ are therefore very sensitive to subtle changes in the torsional features of this triene. The similarity in the intensity of the LTB₄-associated CD bands observed with the receptor refolded in the different surfactants is thus direct evidence that the structural features of BLT1-bound LTB₄ are the same. This is strongly indicative of a similarity in the three-dimensional structural features of the receptor refolded in the different surfactants.

The increase in the intensity of the LTB₄-associated CD bands can be used to monitor the binding of LTB₄ to BLT1, as well as the competition between a BLT1-specific antagonist molecule, U-75302, and LTB₄ for binding to BLT1 [14]. We next measured the respective *K_d* and *K_i* values obtained from the CD-monitored with BLT1 refolded in C₁₃U₉, C₁₃U₁₉, C₁₅U₂₅ or C₁₇U₁₆ or fos-choline-16. As shown in Table 2, these different surfactants stabilize very similar functional states of the receptor. These ligand binding properties of the refolded receptor in the C_{*n*}U_{*m*} surfactants are also closely related to those determined for the receptor expressed in eukaryotic cell [15] indicating that, in all the surfactants used here, the recombinant BLT1 receptor is likely to adopt its native fold. It is to be noted that the receptor refolded in C₁₃U₉ bound its ligands with affinity values closely related to those measured for the receptor in the other C_{*n*}U_{*m*} surfactants, although being present in the solution as a mixture of monomer and dimer. This is consistent with our previous observation that receptor dimerization has no significant effect on the binding of the ligands to the purified BLT1 receptor [27].

We finally analyzed the time-dependent stability of BLT1 refolded in the different detergents. To this end, we followed the ligand-competent receptor fraction as a function of time. The ligand-binding properties of the BLT1 receptor were assessed by circular dichroism, as described above. Fig. 5 shows the percent of active receptor as a function of time. When the receptor was refolded in fos-choline-16 or LDAO, a significant decrease in the functional fraction was observed after a 10-day period so that, after about 20 days, only 50% of the receptor was able to bind the LTB₄ agonist with high affinity. A similar behavior was observed when the receptor was refolded in either DDM/asolectin or HDM/asolectin mixtures (not shown). The decrease in the amount of active receptor probably reflects the unfolding of the receptor under the conditions used. In agreement with this hypothesis, a concomitant decrease in the molar ellipticity at 222 nm in the CD spectrum of the receptor was observed, as shown in the inset to Fig. 5 for the receptor in LDAO or C₁₅U₂₅ (a similar behavior was observed

Table 2
Ligand binding properties of the refolded receptor

Surfactants	C ₁₃ U ₉	C ₁₃ U ₁₉	C ₁₅ U ₂₅	C ₁₇ U ₁₆	fos-choline C16
<i>K_d</i> LTB ₄ (nM)	13.9 ± 2.2	12.5 ± 1.8	13.6 ± 2.1	12.1 ± 1.6	13.1 ± 1.3
<i>K_i</i> U75302 (nM)	217 ± 16	212 ± 12	203 ± 15	219 ± 11	208 ± 17

These values were determined from the CD-monitored titration plots as described in Section 2. The error values correspond to the standard deviation from the mean value calculated from three experiments.

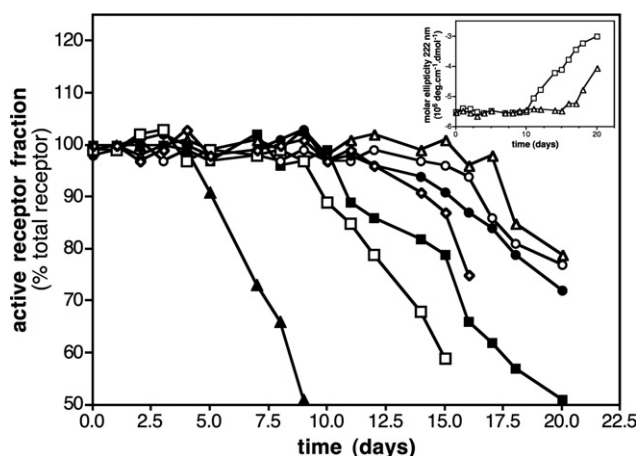


Fig. 5. Time-dependent ligand-binding properties of BLT1. The amount of ligand-competent receptor refolded in the different surfactants was assessed by circular dichroism as a function of time (see Section 2). All the curves correspond to refolding in the presence of the surfactant and aolectins, with the exception of the open diamonds that correspond to the refolding in the presence of only $C_{15}U_{25}$ without aolectins. Closed triangles: $C_{13}U_9$; open squares: LDAO; closed squares: fos-choline-16; closed circles: $C_{13}U_{19}$; open circles: $C_{17}U_{16}$; open triangles: $C_{15}U_{25}$. Inset: variations in the intensity at 222 nm in the CD spectrum of the receptor refolded in either LDAO (open squares) or $C_{15}U_{25}$ (open triangles) as a function of time.

with the other surfactants; not shown). When the receptor was refolded with $C_{13}U_9$ as a surfactant, receptor unfolding started as soon as 5–6 days. In contrast, reconstituting the BLT1 receptor in $C_{13}U_{19}$, $C_{17}U_{16}$ or $C_{15}U_{25}$ allowed a significant increase in its time-dependent stability. In particular, when reconstituted in $C_{15}U_{25}$, BLT1 was stable with no significant unfolding up to 16–17 days, as compared to 10–12 days in the case of fos-choline-16 or LDAO. This shows that refolding of the BLT1 receptor in this surfactant significantly increases its time-dependent stability with no change in either its functional or structural properties.

The data reported here therefore clearly establish that the $C_{13}U_{19}$, $C_{17}U_{16}$ or $C_{15}U_{25}$ surfactants can be favorably used to increase the stability of the native fold of the recombinant receptor BLT1. Moreover, as clearly stated in Table 2, light scattering measurements with the refolded receptors in any of these surfactants clearly indicate that the purified receptor under these conditions is monodisperse with detergent:lipid:protein complexes of well-defined size (the light scattering experiments were carried out after 5 days of storage, at a point where no loss of function of the receptor was observed; see Fig. 5). The absence of polydispersity could be an interesting feature in a crystallization perspective.

It is to be noted that a direct comparison of the effects of the length of the alkyl chains and the nature of the polar head-group of the different surfactants on BLT1 stability can be carried out since they all display cmc values in the same range. The surfactants we produced display the general features of phospholipids with two hydrophobic tails grafted on a polar head but still make micelles as detergents do. Although not affecting the global refolding efficiency, these C_nU_m surfactants stabilize the functional state of the purified receptor better than any of the common detergents we tested so far. Such a result therefore validates our starting hypothesis postulating that phospholipid-like surfactants could associate with membrane

proteins and maintain them soluble in aqueous solution without reduced unfolding better than common detergents.

Besides the length of the alkyl chain that seems to play an important role on receptor stability [14], the data obtained with the different surfactants we produced indicate that, above a certain length of the alkyl chain, the nature of the hydrophilic head could also contribute to the stabilization of the membrane protein in its native fold in solution. Indeed, a significant increase in the stability of BLT1 was achieved with $C_{13}U_{19}$ compared to what was observed with $C_{13}U_9$. Similarly, a slightly higher stability was achieved in $C_{15}U_{25}$ compared to what was obtained with $C_{17}U_{16}$ even if the alkyl chain of the latter is longer than that of the former. The reason for such a difference is still unclear. It is to be noted that the geometrical features of the micelle, in particular its size, are affected by the nature of the polar head (see Table 1). The difference is particularly striking between $C_{13}U_9$ and $C_{13}U_{19}$. If this is the reason for the increased stability of the recombinant BLT1 is an open question.

It is to be noted that the surfactants that lead to an increased stability of the refolded BLT1 also favor the dimeric state of the receptor, whereas $C_{13}U_9$, that is a poor surfactant in the context of BLT1 stability, also leads to the occurrence of mixtures of monomeric and dimeric receptors. However, a direct relationship between dimerization and stability of BLT1 in solution cannot be directly drawn since in fos-choline C-16 the receptor is also essentially dimeric but less stable than in $C_{15}U_{25}$. Therefore, although stabilization of the dimer may contribute to the global stability of BLT1, it is not likely to be the driving force for such stabilizing effects.

Stabilizing the native fold of G protein-coupled receptors in the context of structural studies is of crucial importance if we consider the dramatic lack of structural information on these receptors that are considered so far as the most important targets in the context of drug discovery [28]. Although we and others have developed during the last years an approach that allows the production of GPCRs in high yields through *in vitro* refolding of bacterially expressed proteins [14,29,30], stabilizing the refolded receptor in a native conformation in solution is still a problem. On the basis of the results obtained with the BLT1 receptor, the new surfactant molecules described in this work appear as promising tools to circumvent such a problem.

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